Over-expression of strawberry D-galacturonic acid reductase in potato leads to accumulation of vitamin C with enhanced abiotic stress tolerance

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ABSTRACT

Vitamin C (ascorbic acid) is an essential component for collagen biosynthesis and also for the proper functioning of the cardiovascular system in humans. Unlike most of the animals, humans lack the ability to synthesize ascorbic acid on their own due to a mutation in the gene encoding the last enzyme of ascorbate biosynthesis. As a result, vitamin C must be obtained from dietary sources like plants. In this study, we have developed transgenic potato plants (Solanum tuberosum L. cv. Taedong Valley) over-expressing strawberry GalUR gene under the control of CaMV 35S promoter with increased ascorbic acid levels. Integration of the GalUR gene in the plant genome was confirmed by PCR and Southern blotting. Ascorbic acid (AsA) levels in transgenic tubers were determined by high-performance liquid chromatography (HPLC). The over-expression of GalUR resulted in 1.6–2-fold increase in AsA in transgenic potato and the levels of AsA were positively correlated with increased GalUR activity. The transgenic lines with enhanced vitamin C content showed enhanced tolerance to abiotic stresses induced by methyl viologen (MV), NaCl or mannitol as compared to untransformed control plants. The leaf disc senescence assay showed better tolerance in transgenic lines by retaining higher chlorophyll as compared to the untransformed control plants. Present study demonstrated that the over-expression of GalUR gene enhanced the level of AsA in potato tubers and these transgenics performed better under different abiotic stresses as compared to untransformed control.

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1. Introduction

Vitamin C (L-ascorbic acid, AsA) is an important secondary metabolite of plants that functions as an antioxidant and a cell signaling modulator in a wide array of crucial physiological processes including cell division, growth regulation, and senescence [1–4]. Additionally, ascorbate is a cofactor for many enzymes and affects the expression of genes involved in defense and hormone signaling pathways [5,6]. During evolution, humans have lost the ability to synthesize AsA by a mutation in gene encoding L-gulono-γ-lactone oxidase enzyme responsible for vitamin C synthesis. Thus the dietary plants are the only source for vitamin C. The two proposed biosynthetic pathways of AsA in the plants has

been shown in Fig. 1 [7,8]. The D-mannose/L-galactose or the Smirnoff–Wheeler pathway proceeds via GDP-D-mannose, GDP-L-galactose, L-galactose, and L-galactono-1,4-lactone contributing to the majority of the AsA pool in plants. Although Smirnoff–Wheeler pathway appears to function as the major physiological route of the AsA biosynthesis but the AsA is also synthesized via uronic acid intermediates, such as D-galacturonic acid [8]. In this pathway D-galacturonic acid is reduced to L-galactonic acid by GalUR enzyme, which is subsequently converted to L-galactono-1,4-lactone. The L-galactono-1,4-lactone is further oxidized to AsA by L-galactono-1,4-lactone dehydrogenase (GALDH) enzyme. However, in animals the terminal step of L-gulono-1,4-lactone conversion to AsA is catalyzed by L-gulono-1,4-lactone oxidase (GUO) enzyme [9].
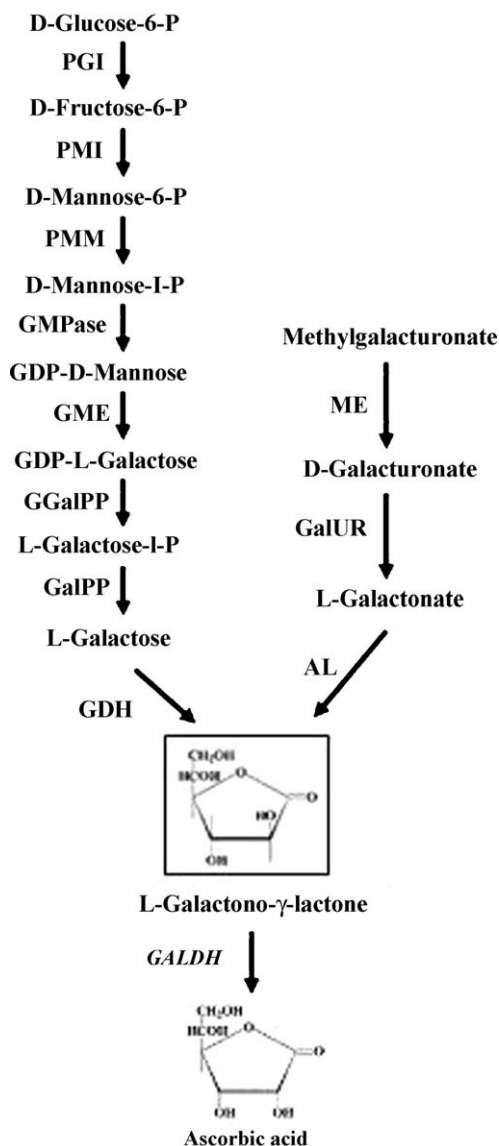
There are several reports where the positive correlation between the activities of the two terminal enzymes of the mannose/galactose pathway, i.e., L-galactose dehydrogenase (GDH), L-galactono-1,4-lactone dehydrogenase (GALDH) and AsA levels in plants has been established [10–13]. This led to concentrated efforts for targeting these enzymes for manipulation

Fig. 1. Proposed pathways for ascorbic acid biosynthesis in plants [7,8]. PGI: phosphogluco isomerase, PMI: phosphomanno isomerase, PMM: phosphomanno mutase, GMPase: GDP-D-mannose pyrophosphorylase, GME: GDP-mannose-3,5-epimerase, GGalPP: GDP-L-galactose pyrophosphatase, GalPP: L-galactose-1-phosphate phosphatase, GDH: L-galactose dehydrogenase, GALDH: L-galactono-1,4-lactone dehydrogenase, ME: methylesterase, GalUR: D-galacturonic acid reductase, AL: aldono-lactonase.

of vitamin C biosynthesis in plants. However, most attempts to enhance AsA levels in plants via manipulation of GALDH and GDH resulted little or no success. The first successful report of enhanced vitamin C content in lettuce and tobacco was achieved by expressing a rat cDNA encoding L-gulono-1,4-lactone oxidase (GUO) [14] where 4–7 folds increase of the AsA level in leaf tissues of lettuce and tobacco were observed. The GalUR gene isolated from strawberry encodes D-galacturonate reductase catalyzes the conversion of D-galacturonic acid to L-galactonic acid and subsequently converted to L-galactono-1,4-lactone which is oxidized to AsA by GDH. Cloning of this gene from strawberry (Fragaria × ananassa Duch.) fruit and its expression in Arabidopsis provided a molecular evidence for D-galacturonic acid as a precursor for vitamin C production [8]. It was also reported that over-expression of GalUR in transgenic A. thaliana resulted 2–3 folds increase in AsA content. Recently, over-expression of an enzyme from the myo-inositol pathway, myo-inositol oxygenase (MIOX4) led to ca. 3 folds increase in AsA content in Arabidopsis [15]. These results obtained in model systems like Arabidopsis and tobacco can be translated into viable strategies for enhancing vitamin C content of nutritionally important crops.

Potato (Solanum tuberosum L.) is an important vegetable crop which ranks fourth among the staple foods of mankind after wheat, rice and maize. Potato production worldwide stands at 293 million tons, of which 36% in developing countries, and covers more than 18 million hectares [16]. Potato being highly nutritious is an excellent target for nutritional improvement. Because of high yield and low cost of cultivation, potato forms an important part of human diet and the nutritional improvement of potato would certainly have a significant impact on human health. Though several earlier studies have demonstrated the possibility of producing transgenics in different plant species with enhanced vitamin C contents, the accumulation of high levels of AsA using GalUR gene in underground storage organs has not yet been achieved. In this study, we report the over-expression of D-galacturonic acid reductase (GalUR) gene from strawberry for enhanced AsA production in potato and evaluation of the transgenic plants for their enhanced tolerance to various abiotic stresses.

2. Materials and methods

2.1. Plant material and growth conditions

Potato cultivar 'Taedong Valley' propagated in culture tubes (25 mm × 150 mm) containing semi-solid basal MS [17] medium supplemented with 30 g l^{-1} sucrose. The pH of the medium was set to 5.7 and gelled with 8 g l^{-1} agar (Duchefa, Germany). The cultures were maintained in growth chamber at 22 ± 2 °C and 16 h light period with an intensity of 100 μmol m^{-2} s^{-1} provided by white fluorescent lamps. Four-week-old in vitro rooted shoots were planted in pots (10 cm in size) containing biopeat (Seminis Asia Ltd., Korea) and transferred to greenhouse for hardening. Young expanding leaves collected from greenhouse hardened plants were used as explants for transformation study. Leaves were surface sterilized with 10% sodium hypochlorite for 15 min followed by three washes with sterile distilled water in laminar flow.

2.2. Bacterial strain and vectors

Transformation was carried out using Agrobacterium tumefaciens strain EHA 105 harbouring the pCAMBIA2300 binary vector. The plasmid vector was kindly provided by Dr. H.J. Hak (Nong Woo Bio., Ltd., Korea). The T-DNA region of the plasmid vector contains GalUR and nptII genes driven by CaMV 35S promoter.

2.3. Agrobacterium-mediated transformation and selection of transgenic plants

A single Agrobacterial colony was cultured in 5 ml of YEP (Yeast Extract Peptone, Duchefa, Germany) medium containing kanamycin (50 mg l^{-1}). The over night grown culture (1 ml) was again inoculated in 50 ml YEP medium containing 50 mg l^{-1} kanamycin and cultured at 28 °C/200 rpm till the OD reached to 0.6 at 650 nm. The bacteria were pelleted and resuspended in MS liquid medium supplemented with 75 μM of acetosyringone (Sigma, MO, USA). This bacterial suspension was used for co-cultivation.

Fully expanded mature leaves collected from 3 to 4 week old potato plants were used for transformation. The leaf tips and basal portions, including the petiole were discarded. The remaining leaf parts were cut into pieces (5 mm × 5 mm) and immersed into the bacterial suspension for 10–20 min. The leaves were then slightly

blotted on sterile filter paper and placed upside down on the co-cultivation medium (MS basal medium) for 2 days at $22 \pm 2\,°C$. After co-cultivation, the explants were kept on callus induction medium (CIM) composed of MS medium supplemented with 1-naphthaleneacetic acid (NAA, 1.0 mg l^{-1}) and Zeatin (1.0 mg l^{-1}). The greenish callus was observed after ca. 20 days from the explants. These callus were transferred to shoot induction medium containing Zeatin (2 mg l^{-1}), NAA (0.01 mg l^{-1}), Gibberellic acid (GA$_3$, 0.1 mg l^{-1}) as well as antibiotics kanamycin (50 mg l^{-1}) and carbenicillin (500 mg l^{-1}) for the selection of transformants. The callus cultures were subcultured to fresh medium on every 10th day. The regenerated shoots (2 cm in length) were excised from callus and transferred to MS basal medium supplemented with kanamycin (50 mg l^{-1}) and carbenicillin (300 mg l^{-1}) for rooting. Rooted plantlets were transferred to pots (25 cm in size) containing biopeat and maintained in greenhouse for tuber formation. These plants were referred as T_0 transgenic lines. The putative T_0 transformants were screened by PCR for the presence of GalUR gene using gene specific primers. The efficiency of transformation was calculated as the number of PCR positive shoots survived on selection medium to the total number of explants kept on co-cultivation medium.

2.4. Raising of T_1 transgenics

The transgenic and untransformed (T_0) control plants were grown in pots (25 cm in size) under greenhouse condition at day temperature of $22\,°C$ and night at $15\,°C$ for tuber formation. The tubers (T_0) collected from 12 weeks old transgenic and untransformed control plants were rinsed with water to remove the soil, dried and kept at room temperature for sprouting. The sprouted T_0 tubers were planted in the pots (25 cm) containing commercial biopeat for obtaining T_1 plants as well as tubers. The plants were watered at every 2-week intervals. The T_1 plants and tubers were taken for the molecular, biochemical and physiological analyses. Single node cuttings from T_1 transgenic lines were taken for the *in vitro* stress analyses.

2.5. DNA isolation and PCR analysis

Genomic DNA was isolated from 0.5 g of young leaves by the CTAB method [18]. The PCR amplification was carried out using the gene specific primers (5′-ATG GCA AAG GTT CCT TCA GTA-3′) and (5′-TCA TAA TTC TTC GTC AAC TTC-3′) for GalUR and (5′-CCA TGA TA TTC GGC AAG CAG GCA T-3′) and (5′-ACAAGATGGATTGCACG-CAGGTTC-3′) for nptII. PCR was performed in a 25 μl reaction mixture containing 50 ng of DNA as template, 1× Taq DNA polymerase buffer, 400 μM each dNTPs, 10 pmol of each oligo-nucleotide primer and 0.3 U of Taq DNA polymerase (Takara, Japan). DNA amplifications were done in a thermal cycler (Takara, Japan) using the following program: initial denaturation at $94\,°C$ for 5 min, followed by 35 cycles of denaturation at $94\,°C$ for 1 min, annealing at $55\,°C$ for GalUR and $56\,°C$ for nptII for 1 min and extension at $72\,°C$ for 2 min followed by final extension for 7 min at $72\,°C$. The amplification products were visualized on 1% (w/v) agarose gel stained with ethidium bromide (0.5 μg/ml) under UV light.

2.6. Southern blot

Southern blot [19] analysis was performed according to the standard procedure [20]. Approximately 20 μg of genomic DNA isolated from transgenic and untransformed plants were used for Southern blotting. DNA was double digested with BamHI and KpnI to confirm the presence of inserted GalUR gene and single digestion with BamHI to detect the copy number as well as integration of the gene. The digested DNA was electrophoresed on 1% agarose gel and

transferred onto Hybond-N+ nylon membrane (Roche Co., Germany) by capillary blotting method. Dig-labelled GalUR probe was generated by PCR Dig Labelling Mix (Roche Co., Germany) with the above mentioned primers. After overnight hybridization, the membrane was washed with 2× SSC containing 0.1% SDS at $58\,°C$ for 5 min and 0.2× SSC containing 0.1% SDS at $58\,°C$ for 10 min. The membrane was then detected using the Dig Detection kit according to the manufacturer's instructions (Roche Co., Germany).

2.7. Reverse transcriptase-PCR (RT-PCR)

Total RNA was isolated from the transgenic as well as from untransformed control plants using TRI reagent (Sigma, USA). 5 μg of total RNA was taken in a nuclease free tube. The OligodT primer (3 μl of 100 ng/μl) and DEPC treated water were added to it so that the final volume was made up to 50 μl. This mixture was incubated at $65\,°C$ for 5 min and then slowly cooled to RT so that the oligodTs bind to polyA tail. 5 μl of 10× SuperScript^{TM} Reverse Transcriptase buffer (Invitrogen, USA) was added along with 1 μl RNase inhibitor (40 °U/μl), 2 μl 100 mM dNTP mix and 1–2 μl SuperScript^{TM} Reverse Transcriptase enzyme. The enzyme mixture was gently mixed and incubated at $42\,°C$ for 1 h. The enzyme was deactivated by heating the reaction mixture at $90\,°C$ for 5 min. The cDNA (1 μl) synthesized from the transgenic and untransformed control lines were taken as template for PCR using the GaUR gene specific primers with same PCR condition. Actin mRNA served as loading control. Reaction products were separated on 1% agarose gel stained with ethidium bromide and visualized under UV light.

2.8. Quantitative RT-PCR

Total RNA was extracted and treated with DNase I before using for reverse transcription, using random hexamers as primers and SuperScript-II reverse transcriptase (Invitrogen, USA), to generate a first strand cDNA template. Samples were amplified using ABI Prism 7700 sequence detector (Applied Biosystems). The real-time PCR amplification of GalUR gene (144 bp) was carried out using cDNA specific primers (5′-CTGTAATCGGCATGGGAACT-3′ and 5′-TGGCTTCACCGAGATCTTTC-3′). The PCR was performed using SYBR green PCR kit (Qiagen GmbH, Hilden, Germany). Actin was used as an internal control. PCR conditions were as follows: 5 min initial denaturation at $95\,°C$ followed by 30 cycles of denaturation at $94\,°C$ for 30 s, annealing at $50\,°C$ for 30 s and an extension at $72\,°C$ for 2 min. Comparative threshold (C_t) values were normalized to actin control and compared to obtain relative expression levels.

2.9. Extraction and estimation of ascorbic acid (AsA) by HPLC

Extraction of ascorbic acid from Southern positive T_1 potato plant tubers were performed as described [21]. Tubers from the lower 2/3 portion of the pot were collected separately from superficial ones, washed in water, dried briefly at room temperature, peeled, cut in to pieces and frozen at $-80\,°C$ prior to analysis. Approximately 2 g of frozen tubers were grind in 5% metaphosphoric acid using a commercial blender. The mixture was filtered through whatman no. 1 filter paper and the residue was rinsed three times with 5 ml of 5% metaphosphoric acid. The filtrates were centrifuged at $18,000 \times g$ for 10 min at $1\,°C$. The samples were diluted to 25 ml with 5% metaphosphoric acid and used for HPLC analysis.

HPLC was carried out on Agilent 1100 HPLC system (Agilent Technologies, Inc., Waldbronn, Germany) using an Atlantis® dC18 column (4.6 mm × 250 mm, 5 μm pore size). The flow rate used was 3 ml/min with a mobile phase of 0.2% TFA (A) and 100%

methanol (B). The samples were filtered through 0.45 μm syringe filter (Toyo Roshi Kaisha, Ltd., Japan) and 20 μl sample was injected to the HPLC system. The eluants were monitored with a UV detector at 254 nm. Three separate analyses were carried out with each sample and the AsA content was expressed as μmol/g FW.

2.10. D-Galacturonic acid reductase (GalUR) assay

Protein extraction from Southern positive T_1 potato tubers was carried out as described [22]. One gram of tuber samples were homogenized in liquid nitrogen and extracted with 20 mM MOPS buffer, pH 6.9, containing 1 mM $MgCl_2$, 2 mM β-mercaptoethanol, 3 mM EDTA, 1 mM EGTA, 5% glycerol, 0.25 M sucrose and protease inhibitor (1 mM PMSF) (2 ml buffer/g of fresh tissue). The suspensions were centrifuged for 30 min at $27,000 \times g$ at 4 °C. Supernatant were taken for enzyme assay. Protein content of the crude extract was quantified by the protein dye binding assay with bovine serum albumin as the standard [23].

D-Galacturonic acid reductase (GalUR) activity was determined as described in [8]. GalUR activity was measured by the decrease in absorbance at 340 nm at 25 °C after the addition of 100 μl of crude enzyme extract to the assay medium (1 ml) consisted of 50 mM phosphate buffer (pH 7.2), 2 mM EDTA, 0.1 mM NADPH, 30 mM D-galacturonic acid and 2 mM DTT. The GalUR activity in the crude enzyme extract was expressed as nmol of NADPH oxidized min^{-1} mg^{-1} protein.

2.11. Substrate feeding assay

Standard protocol [13] was used for the substrate feeding assay. Fully expanded young leaves from Southern positive T_1 lines as well as untransformed control potato plants were taken. The leaves were kept in 100 ml flasks containing freshly prepared 30 mM solution of D-Galacturonic acid (D-GAL). Distilled water was used as control. Leaves were maintained at 22 °C under a 16 h photoperiod. The leaves were transferred to freshly prepared substrate solutions at every 12 h and the samples were collected after 72 h for AsA analysis by HPLC. Three independent analyses were carried out for each sample.

2.12. Physiological analysis for abiotic stress tolerance

2.12.1. Leaf disc assay
Leaf disc assay were carried out to evaluate the sensitivity of the transformed and untransformed potato plants to methyl viologen (MV), sodium chloride (NaCl) and mannitol stresses as described [24]. The leaf discs (1.0 cm^2) were excised from healthy and fully expanded potato leaves of 6–8-week-old transgenics (T_1) and untransformed control plants using a cork borer. Leaf discs were floated in 5 ml solutions of MV at concentrations of DW, 2, 5, 10 μM, and NaCl (DW, 200, 400, 600 mM) as well as mannitol at concentrations of DW, 100, 200, 300 mM for 4 days. The leaf discs were incubated under continuous white light of 100 μmol m^{-2} s^{-1} intensity at 22 ± 2 °C. The effect of various treatments on leaf discs was observed by monitoring the phenotypic changes and by measuring the chlorophyll content.

2.12.2. Determination of the chlorophyll content
The chlorophyll content in the leaf discs treated with MV, NaCl and mannitol were estimated as described [25]. The leaf discs were homogenized thoroughly in 1 ml of 80% acetone and the homogenate was centrifuged at 3500 rpm for 3 min. The supernatant was retained and the absorbance was recorded at 663 and 646 nm. The chlorophyll content was expressed in mg per gram fresh weight.

2.12.3. In vitro evaluation for salt tolerance
The Southern positive transgenic (T_1) lines $T_1^{1.4}$ and $T_1^{9.3}$ as well as untransformed control shoots were grown on MS medium supplemented with NaCl. Salt stress was accomplished by transferring single node segments to the culture tubes containing MS basal medium supplemented with different concentrations of NaCl (0, 50, 100, 150, and 200 mg l^{-1}). Plants were cultured in a growth chamber under a 16 h photoperiod with a light intensity of 100 μM m^{-2} s^{-1} at 20 ± 2 °C. Tolerance to salt stress was estimated by measuring the shoot and root length after 30 days of growth.

2.13. Statistical analysis

Data collected from different experiments were analyzed using Statistical Analysis Software (SAS Inc., USA) package 9.1. Statistically significant differences between means were determined by Duncan's multiple range test at P (<0.01) level.

3. Results

### 3.1. Agrobacterium mediated transformation and recovery of transgenics

Transgenic potato plants were developed by the over-expression of GalUR gene via Agrobacterium-mediated transformation. The expression of GalUR gene was driven by CaMV 35S promoter. Regeneration of the shoots were observed from the callus formed from the leaf sections after 4 weeks on shoot induction medium. Efficient shoot development was achieved on shoot induction medium supplemented with Zeatin (2 mg l^{-1}), NAA (0.01 mg l^{-1}) and GA_3 (0.1 mg l^{-1}). Elongated shoots (ca. 2 cm in length) were transferred to rooting medium (MS basal) supplemented with 50 mg l^{-1} kanamycin. The roots were observed after 2–3 weeks. Well rooted plantlets were transferred to green house for hardening and further growth. From 120 explants (taken from four different experiments) inoculated with Agrobacterium, a total of 21 shoots were obtained on selection medium. Of these, 16 shoots developed roots on rooting medium containing kanamycin. Subsequently 16 shoots were transferred to pots where 13 of them survived. In the PCR analysis all the 13 plants were found to be PCR positive for GalUR gene (data not shown). The transformation efficiency was found to be 10.4%. The T_0 tubers were harvested after 12 weeks and kept at room temperature for sprouting. The tubers (T_0) from seven PCR positive lines were selected and planted for generation of T_1 transgenic plants. However, only three lines were able to sprout and produce T_1 plants and subsequently formed tubers. All the transgenic plants were phenotypically uniform and undistinguishable from the untransformed control plants.

3.2. Molecular analysis of T_1 transgenic lines

The three T_1 transgenic lines ($T_1^{1.4}$, $T_1^{6.1}$ and $T_1^{9.3}$) were found to be PCR positive showing the amplification of 1 kb band on agarose gel corresponding to GalUR and 584 bp band for nptII genes, whereas no amplification was observed in the untransformed control plants.

Southern blot analyses were carried out to know the integration of gene as well as the estimated copy number of transgene integrated. The estimated copy number of the GalUR gene inserted in different transgenic lines ranged from one to two. Of the three tested T_1 transgenic lines, two lines ($T_1^{1.4}$ and $T_1^{6.1}$) tested contained a single gene copy, while $T_1^{9.3}$ had two integrated copies (Fig. 2a). The variable size of the gene on the blot indicated independent integration events in different transgenic plants. The Southern blot performed to check the transgene integration
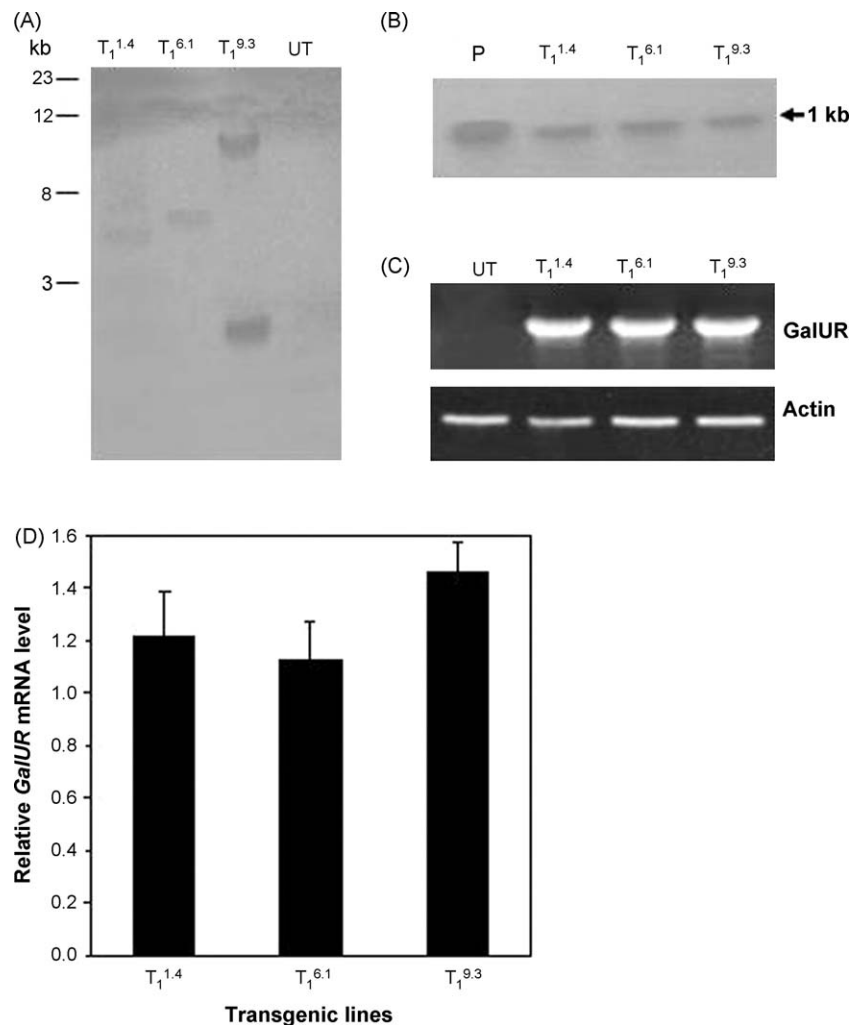
Fig. 2. (a) Southern blot analysis of different independent T_1 potato transgenic lines showing copy number and (b) the gene integration. Lane UT: untransformed control; P: plasmid DNA having the *GalUR* gene. (c) RT-PCR analysis of T_1 transgenic lines using *GalUR* gene specific primers. Lane UT: untransformed plants; lanes $T_1^{1.4}$–$T_1^{9.3}$: the transgenic lines. The actin gene fragment (600 bp) was amplified in RT-PCR as an internal control. (d) The relative *GalUR* expression levels of $T_1^{1.4}$, $T_1^{6.1}$ and $T_1^{9.3}$ potato lines. The comparative threshold values were normalized with actin internal control and plotted. Each line represents mean ± SD of three independent measurements.

showed the inserted loci of the transgene in the potato genome (Fig. 2b). No hybridization signal was detected in untransformed control plants.

Expression levels of *GalUR* were determined in the T_1 transgenic lines through Reverse Transcriptase-PCR (RT-PCR). As shown in Fig. 2c, an expected 1 kb amplification corresponding to *GalUR* gene was detected in all the three transgenic lines, where as no amplification was detected in the untransformed control plants. The RT-PCR result displayed increased *GalUR* mRNA expression in the transgenic lines when compared to actin control. However, notable variations in the intensity of the bands between the transgenic lines were not detected.

Therefore, to further quantify the mRNA expression levels between transgenic lines, we carried out quantitative Real-Time PCR (RT-PCR), the most sensitive method for detection of accurate mRNA expression level. The *GalUR* mRNA expression level was related to the expression level of internal control actin gene. The data shown here is for three replicates of each sample. Relatively higher *GalUR* mRNA expression levels were detected in all the transgenic lines (Fig. 2d). However, the transgenic line ($T_1^{9.3}$) with two copies of transgene (as evident from the Southern analysis) showed slightly higher *GalUR* expression level as compared to the transgenic lines ($T_1^{1.4}$ and $T_1^{6.1}$) with a single copy of transgene.

3.3. Biochemical analysis of T_1 transgenic lines

The activity of GalUR was measured by spectrophotometric method to detect the oxidation of NADPH. The Southern positive T_1 lines ($T_1^{1.4}$, $T_1^{6.1}$ and $T_1^{9.3}$) showed 2–2.5 folds higher GalUR activity (Fig. 3a) as compared to the untransformed control lines.

Leaves from *GalUR* transgenic potato plants fed with D-GAL contained 11–13 folds higher AsA content as compared to the transgenic leaves kept in distilled water (Table 1).

3.4. Analysis of AsA content in T_1 transgenic tubers by HPLC

The Southern positive T_1 tuber samples collected from all the individual transgenic and untransformed plants were analyzed for AsA levels by HPLC. Transgenic tubers ($T_1^{1.4}$, $T_1^{6.1}$ and $T_1^{9.3}$) accumulated up to 2.8, 2.52 and 3.2 μmol/g FW AsA content, respectively representing a 1.6–2-fold increase in AsA over non-transformed tubers (Fig. 3b). All the transgenic tubers harbouring *GalUR* had significantly higher levels of AsA than that of untransformed tubers. These results indicate that *GalUR* gene driven under the control of *CaMV* 35S promoter was stably expressed in increasing the AsA contents in potato tubers.
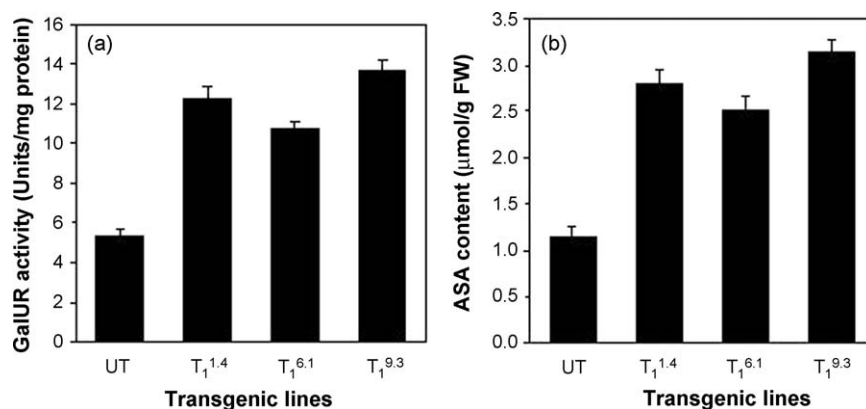
Fig. 3. GalUR activity and AsA contents in T_1 transgenic and untransformed (UT) control lines. (a) The GalUR activity of T_1 transgenic ($T_1^{1.4}$, $T_1^{6.1}$ and $T_1^{9.3}$) and untransformed (UT) control lines. Data represents the means ± SD of three replicates. (b) The AsA contents of three potato plants ($T_1^{1.4}$, $T_1^{6.1}$ and $T_1^{9.3}$). Each value represents the mean ± SD of three independent measurements.

Table 1
Effect of precursor feeding on AsA content of T_1 transgenics ($T_1^{1.4}$, $T_1^{6.1}$ and $T_1^{9.3}$) and untransformed (UT) control lines.

Treatments	AsA content (μmol g^{-1} FW)[b]			
	UT	$T_1^{1.4}$	$T_1^{6.1}$	$T_1^{9.3}$
Distilled water (DW)	0.09 ± 0.002	0.23 ± 0.016b	0.17 ± 0.025b	0.27 ± 0.033b
D-Galacturonic acid (D-GAL)	0.46 ± 0.163	2.52 ± 0.521a	2.05 ± 1.032a	3.53 ± 0.752a

[a] Four-week-old leaves fed with 30 mM D-GAL and DW for 72 h.
The alphabet letters (a, b) indicates significant differences ($P < 0.01$) by Duncan's multiple range tests.
  [b] Values represent the mean ± SD of three independent measurements.

3.5. Phenotypic evaluation of transgenic lines for abiotic stresses tolerance

3.5.1. Leaf disc assay

To test whether the over-expression of the *GalUR* gene can alter the sensitivity of transformed plants towards methyl viologen (oxidative stress), NaCl (salt stress) and mannitol (drought stress) stresses leaf disc senescence tests were performed. Leaf discs of equal diameter (1 cm) from fully grown mature leaves of the T_1 transgenic ($T_1^{1.4}$ and $T_1^{9.3}$) and untransformed control lines were floated either on MV (2, 5 and 10 μM), NaCl (200, 400 and 600 mM) and mannitol (100, 200 and 300 mM) as mentioned in Section 2.

Significant differences were observed between the leaf discs derived from untransformed control and transgenic lines after 4 days of MV (10 μM) treatments (Fig. 4a). While the leaf discs from the control lines showed complete senescence, those from the transgenic lines remained green. Measurement of chlorophyll content of the leaf discs (Fig. 4b) confirmed the reason for the phenotypic differences observed. The results revealed that the chlorophyll content retained in the leaf discs of T_1 transgenic lines exposed to 5 and 10 μM methyl viologen stress was approximately 2–3 folds higher as compared to the leaf discs of untransformed control lines.

Similarly the leaf discs of T_1 transgenic lines floated on NaCl (400 and 600 mM) and mannitol (200 and 300 mM) showed delayed senescence. While the leaf discs from untransformed control plants showed complete bleaching at 600 mM NaCl and 300 mM mannitol after 4 days of treatment (Fig. 4c and e), the transgenic leaf discs remained green. Measurement of chlorophyll content showed 2–2.5 folds higher content in the leaf discs from transgenic versus the untransformed control lines (Fig. 4d and f), thus indicating greater tolerance of the former to NaCl stress as compared to the later.

3.5.2. In vitro evaluation for salt tolerance

In vitro grown transgenic shoots on MS medium supplemented with different concentrations of NaCl showed enhanced tolerance to NaCl compared to untransformed control plants (Fig. 5a). NaCl treatment caused significant reduction in the shoot and root length in both transgenic and untransformed. However, transgenic plants grown at a salt concentration (200 mM NaCl) exhibited a significantly higher shoot length (Fig. 5b) and root length (Fig. 5c) as compared to untransformed control lines.

4. Discussion

Notable efforts have been made towards development of transgenic plants with enhanced AsA content. Earlier, successful attempts were reported for increasing AsA content by constitutive expression of *GalUR* gene from strawberry in *Arabidopsis* [8] and tomato hairy roots [26]; over-expression of a myo-inositol oxygenase in *Arabidopsis* [14]; expression of dehydroascorbate reductase (DHAR) from wheat in tobacco and maize [27] and constitutive expression of *GUO* gene in lettuce and tobacco [13]. However, to the best of our knowledge there is no report on metabolic engineering of AsA in potato plants. In our endeavour to enhance vitamin C content in potato, we have over-expressed *GalUR* gene from strawberry under a constitutive promoter, *CaMV 35S*. The transgenic lines (with one and two copies insertions) were phenotypically normal and showed up to 1.6–2-fold increase of vitamin C in tubers. Similar result was reported in maize by the expression of *DHAR*, which resulted in a 2-fold increase in AsA content in non-photosynthetic kernels [27]. In our study, the increased AsA content with higher levels of GalUR enzyme activity was correlated with mRNA expression. The similar finding showing positive correlation between enzyme activity and AsA content was reported in *Arabidopsis* [8]. However, a basal level of the GalUR activity was also observed in the untransformed line (UT). Although
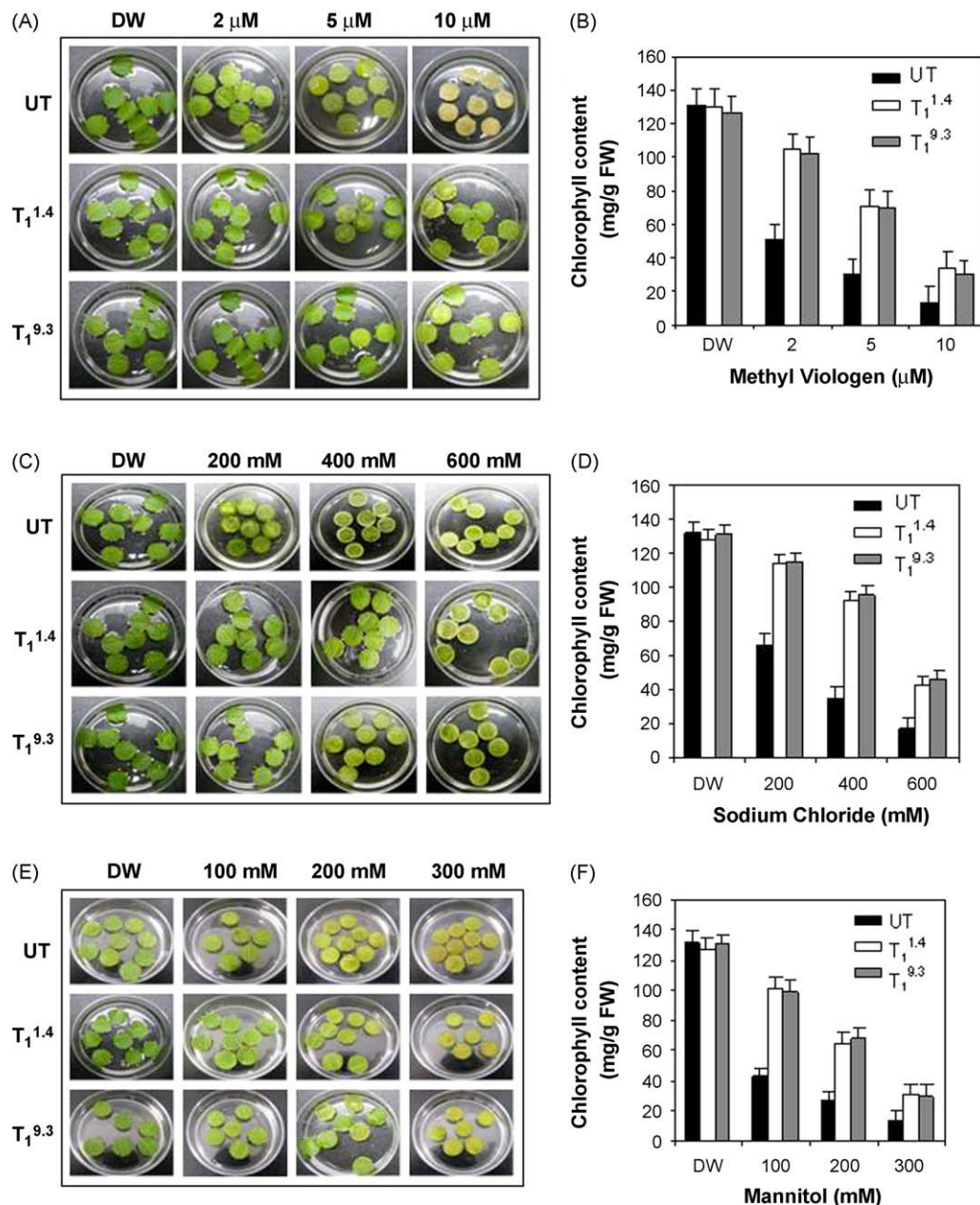
Fig. 4. Retardation of methyl viologen, salt and mannitol stress induced senescence of leaf discs from the transgenic lines of potato over-expressing the *GalUR* gene. (a) Phenotypic differences in the leaf discs from transgenic vs. the untransformed control lines after 4 days of MV treatment. (b) Chlorophyll content (mg g^{-1} fresh weight) of leaf discs of transgenic lines $T_1^{1.4}$ and $T_1^{9.3}$ vs. the untransformed control (UT) line floated on 2, 5 and 10 µM MV solution, respectively, for 4 days under continuous white light at 24 ± 2 °C. (c) Phenotypic differences in the leaf discs from transgenic vs. the untransformed control lines. (d) Chlorophyll content (mg g^{-1} fresh weight) of leaf discs of transgenic lines $T_1^{1.4}$ and $T_1^{9.3}$ vs. the untransformed control (UT) line floated on 200, 400 and 600 mM NaCl solution, respectively, for 4 days under continuous white light at 24 ± 2 °C. (e) Phenotypic differences in the leaf discs from transgenic vs. the untransformed control lines. (f) Chlorophyll content (mg g^{-1} fresh weight) of leaf discs of transgenic lines $T_1^{1.4}$ and $T_1^{9.3}$ vs. the untransformed control (UT) line floated on 100, 200 and 300 mM mannitol solution, respectively, for 4 days under continuous white light at 24 ± 2 °C.

the *GalUR* homologue in the potato gene has not yet been reported; the probable reason for the basal enzyme activity could be the conceivable involvement of the conversion of D-galacturonic acid by a non-specific type of reductase activity. There is a similar report where the D-glucuronolactone reductase, an enzyme from the animal pathway, also exhibited activity with D-galacturonic acid [28] and very recently the metabolic profiling analysis showed the presence of glucuronic and gulonic acids, intermediates of animal AsA biosynthetic pathway in *Arabidopsis* [29].

Recently, a substantial increase in α-tocopherol and AsA contents was reported in potato tubers by over-expression of homogentisate phytyltransferase from *Arabidopsis thaliana* [30]

and dehydroascorbate reductase from sesame hairy root cultures [31], respectively. In both the cases the transgene was driven under the control of *CaMV* 35S promoter. We therefore used *CaMV* 35S promoter to drive *GalUR* gene from strawberry for its strong and constitutive expression in potato tubers.

Different explant types have been used for obtaining transgenics in *S. tuberosum* L. viz. internodes [32] and leaf explants [33] derived from *in vitro* grown plants. Leaf explants obtained from *in vivo* grown 4–6-week-old plants can be easily manipulated as compared to shoot tips and were therefore chosen for further experiments. We observed that the callus initiation from cut edges of the swollen explants occurred within 7–10 days of culture. The

(A)
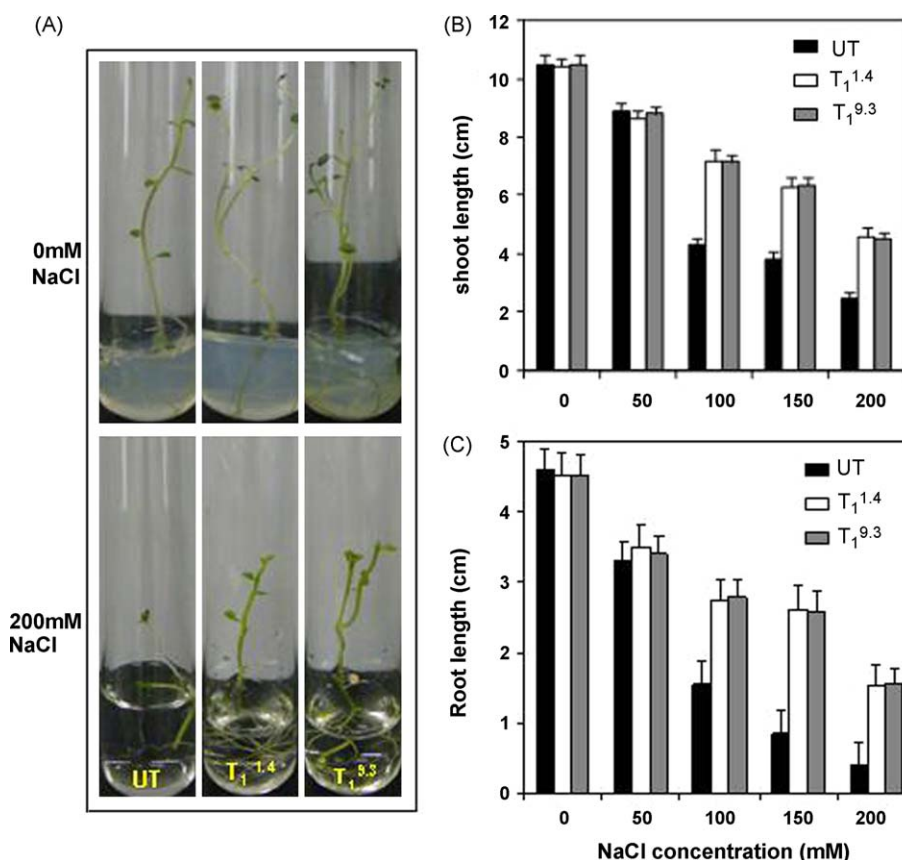
Fig. 5. Effect of salt stress on transgenic lines ($T_1^{1.4}$ and $T_1^{9.3}$) and untransformed (UT) control potato plants. (a) The growth pattern of the transgenics and untransformed control shoots on MS medium supplemented with NaCl. (b) Graph representing the shoot length from of transgenic and untransformed control plants after 30 days under different concentrations of NaCl. (c) The graph representing the root length of the transgenic and untransformed control plants after 30 days. Values are the mean $\pm$ SD of three independent measurements.

callus response exhibited by the leaf explants was better with higher level of NAA (1 mg l^{-1}). NAA was found to be very effective for callus induction and the combination of Zeatin, NAA and GA$_3$ resulted in a substantial increase in shoot regeneration. Presence of NAA (0.01 mg l^{-1}) at low levels in the shoot induction medium helped to maintain shoot growth by controlling excessive callus proliferation, whereas Zeatin and GA$_3$ induced shoot bud regeneration and shoot elongation. The combination of Zeatin riboside (2.2 mg l^{-1}), NAA (0.02 mg l^{-1}) and GA$_3$ (0.5 mg l^{-1}) used for transgenic shoot regeneration from *in vitro* leaf discs of potato (*S. tuberosum* L. ssp. *andigena*) showed a transformation frequency of 35.6% [34].

Increased tolerance of transgenic tobacco with elevated levels of vitamin C to salt and oxidative stresses was reported with the over-expression of *DHAR* [35] and *GALDH* [36], respectively. These reports suggested that increased vitamin C might be responsible for increased tolerance of plants to different abiotic stresses. Vitamin C is reported to be involved in enzymatic or non-enzymatic radical scavenging processes in plants [1]. It has been reported that the increased AsA in transgenic BY-2 cell lines by over-expression of *GALDH* gene imparted resistance to oxidative stress caused by MV [36]. It was also reported that exogeneous AsA increased plant resistance to salt stress, and the increased resistance was associated with the antioxidant activity of the AsA [37]. In our studies, the *GalUR* transgenics with elevated AsA levels displayed enhanced tolerance to various abiotic stresses imposed by methyl viologen (10 μM), NaCl (600 mM) and mannitol (300 mM). There are several reports where the increase in endogeneous level of water soluble antioxidants and/or antioxidants enzymes have been reported under the salt stress

[38–41]. In the present study southern positive transgenic lines showed enhanced tolerance to NaCl (200 mM) with increased shoot and root length as compared to untransformed control lines. Similar results were reported in transgenic tobacco plants with increased AsA levels grown in high salt concentrations [35].

In conclusion, over-expression of *GalUR* gene under *CaMV* 35S constitutive promoter resulted in a 2-fold increase in AsA content in transgenic potato tubers. The increased levels of AsA were correlated with the increased mRNA expression and activity of GalUR enzyme. Transgenic potato plants showed enhanced tolerance to various abiotic stresses caused by MV, NaCl and mannitol under *in vitro* conditions. We thus demonstrated that over-expression of *GalUR* gene under the control of *CaMV* 35S promoter increases vitamin C biosynthesis and also imparted increased tolerance to various abiotic stresses.

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